Independent Induction of Senescence by \( p16^{INK4a} \) and \( p21^{CIP1} \) in Spontaneously Immortalized Human Fibroblasts

Marguerite Vogt, Candy Haggbloom, Jo Yeargin, Trudy Christiansen-Weber, and Martin Haas

Molecular Biology and Virology Laboratory, Salk Institute, San Diego, California 92186-5800 [M. V.; C. H.], and Department of Biology and Cancer Center, University of California, San Diego, La Jolla, California 92039-0063 [J. Y., T. C.-W., M. H.]

Abstract

In this work, we address the question of whether replicative senescence can be induced in immortal nontumorigenic human fibroblasts. The immortal fibroblasts used in this study were derived from two Li-Fraumeni (LF) patients who carry in their germ line one wild-type and one mutant \( p53 \) allele. Both immortal lines have lost the \( \text{wt} p53 \) allele and express no detectable \( p16^{INK4a} \) protein, although they carry the \( p16^{INK4a} \) gene. In contrast to immortal human fibroblasts, senescent human fibroblasts have a low content of 5-methyl-cytosine in their DNA. This observation suggested the possibility that a demethylating agent could revert the immortal phenotype and induce replicative senescence in the immortal cell lines. Cells of the two LF lines were exposed to the demethylating agent 5-aza-2'-deoxycytidine. Within 6 days, all cells were growth arrested and showed the enlarged and flat morphology characteristic of senescent cells, an accumulation of lipofuscin granules and senescence-associated \( \beta \)-galactosidase activity at pH 6, both biomarkers for senescence. Immunoblots of 5-aza-2'-deoxycytidine-treated cells showed a greatly increased expression of \( p16^{INK4a} \) protein but no detectable change in the expression of \( p21^{CIP1} \), a gene known to be strongly expressed in senescent normal human fibroblasts. In two other experimental series, cells of the two LF lines were infected with retroviral constructs encoding either \( p16^{INK4a} \) or \( p21^{CIP1} \). Each of the transduced genes induced senescence without affecting the expression of the other endogenous gene. The results show that induction of senescence in immortal LF fibroblasts can occur by different pathways: (a) by demethylation-dependent pathways that induce the expression of \( p16^{INK4a} \); and (b) by demethylation-independent pathways involving the expression of \( p21^{CIP1} \). The induction of senescence by \( p16^{INK4a} \) and \( p21^{CIP1} \) occurred equally in the two human immortal fibroblast lines, which differed in the length of their telomeres and the activity of their telomerase.

Introduction

Normal human fibroblasts, when cultured in vitro, undergo a finite number of divisions before they senesce (1). It was first suggested by Olovnikov (2) that an important factor in the aging process of human somatic cells is the progressive shortening of the telomeres with each cell doubling, which eventually leads to cell death. It was subsequently confirmed that telomeres shorten with each round of replication until they reach a critical length beyond which no further cell doubling occurs. The telomeres act, therefore, as a "mitotic clock" that determines the time at which replicative senescence occurs (3). Cells that have an indefinite growth potential, so-called immortal cells, escape from replicative senescence by maintaining the length of their telomeres, in most cases due to the reactivation of a telomerase. In studying 35 immortal human cell lines, Bryan et al. (5) found that 20 lines had telomerase activity that could be detected by the telomeric repeat amplification protocol (4) assay, and that the 15 lines without detectable telomerase activity had abnormally long and heterogeneous telomeres. The mechanism by which the 15 lines overcame the shortening of their telomeres and acquired abnormally long telomeres is presently unknown. However, these findings suggest that to acquire an indefinite life span, the telomeres of the cell have to be maintained above a critical length, giving support to the hypothesis that the shortening of telomeres beyond a critical length plays the role of a mitotic clock. It has been suggested that the cell possesses another mitotic clock, a "demethylation clock," that is responsible for cell aging (6). It was shown that the 5-methyl cytosine content of cellular DNA decreases during in vitro passaging of normal fibroblasts, suggesting that demethylation plays a role in cell aging (7). Upon the acquisition of an indefinite growth potential (immortalization), the 5-methylcytosine content increases to the level found in young fibroblasts (7).

The spontaneous acquisition of an indefinite growth potential, an important trait of most cancer cells, occurs rarely in normal human fibroblasts. Two human fibroblast lines with the capacity to divide indefinitely were obtained by Bischoff et al. (8) when culturing normal fibroblasts from two patients with the LF\(^3\) syndrome. The two lines differ in the activity of their telomerase and the lengths of their telomeres. We were interested in whether it was possible to revert the immortal...
phenotype of these two lines into a senescent phenotype. We used three approaches: demethylation by 5-aza-2'-deoxycytidine and infection with retroviral constructs encoding either p16\(_{\text{INK4a}}\) (p16INK4a/CDKN2/MTS1) or p21\(_{\text{CIP1}}\) (p21CIP1/WAF1/sdi1). All three approaches induced complete senescence of the cells in both lines within 6 days.

**Results**

**Properties of Two Spontaneously Immortalized Human Fibroblast Lines MDAHO41 and MDAHO87.** The two immortal human fibroblast lines, MDAHO41 and MDAHO87, were derived from patients with the LF syndrome (8). LF patients, who suffer from a high incidence of spontaneous cancer early in their lives, carry in their germ line one wild-type p53 allele and one mutated p53 allele (8). The two patients and the lines derived from them differ in their p53 mutation. Line MDAHO41 has a deletion in the p53 allele, which leads to a truncated p53 protein; line MDAHO87 expresses a mutated p53 protein due to a missense mutation at codon 248 (CGG→Arg→TGG→Trp) in the DNA-binding domain of p53. MDAHO41 cells express low levels of p21\(_{\text{CIP1}}\) protein (Fig. 1b), whereas no p21\(_{\text{CIP1}}\) protein could be detected in MDAHO87 cells (Fig. 2b). Both immortal lines have lost the wild-type allele of p53 (9) and show no detectable p16\(_{\text{INK4a}}\) protein (Figs. 1a and 2a). The loss of wt p53 and the loss of expression of the tumor suppressor gene p16\(_{\text{INK4a}}\) may have been preconditions enabling the immortalization of the two lines in vitro. It is of interest that another immortal cell line isolated from fibroblasts of a different LF family also showed a loss of both wt p53 and p16\(_{\text{INK4a}}\) expression (10).

Lines MDAHO41 and MDAHO87 differ in their telomerase activity. Using a modified Morin assay (see "Materials and Methods") MDAHO41 cells have a telomerase activity that is slightly lower than that of 293 cells when equal amounts of protein from S-100 extracts are used for the extension reaction (Fig. 3a, Lanes 1 and 4). In contrast, no telomerase activity could be detected with an equal amount of protein from MDAHO87 S-100 extracts (Fig. 3a, Lane 7). The presence of an inhibitor in the extracts from MDAHO87 cells could be excluded because the mixing of an MDAHO87 S-100 extract with an MDAHO41 S-100 extract at a protein ratio 1:1 (compare Lanes 4 and 10) or 70:1 (data not shown) did not affect the telomerase activity of the MDAHO41 cells. Interestingly, a low level of telomerase activity was observed in 087 cells when a 10 times larger amount of protein from the S-100 extract (14 μg of protein corresponding to approximately 3 × 10\(^5\) cells) was used (Fig. 3b, Lane 5). The shorter ladder suggests a lower processivity of the enzyme.

The cells of the two immortal lines MDAHO41 and MDAHO87 differ also in the lengths of their telomeres (Fig. 4). The TRFs obtained after Hinfl and RsaI digestion of genomic DNA of MDAHO41 cells showed a spread between 1.4 and 4.8 kb, which did not change significantly over many transfers (Lanes 1–3). In contrast, the length of the TRFs of MDAHO87 cells was above 20 kb (Lane 4).
Demethylation Induces Senescence in Lines MDAH041 and MDAH087. Wilson and Jones (7) were the first to show that the 5-methyl-cytosine content of cellular DNA of normal diploid fibroblasts from mice, hamsters, and humans decreases significantly with increased time of culturing. It was subsequently found that the in vitro life span of normal human fibroblasts could be shortened by exposure of the cells to the demethylating agents 5-aza-ctydine or 5-aza-CdR (6, 11). These findings lend support to the hypothesis that demethylation of 5-methylcytosine is causally related to certain aspects of in vitro aging (6, 11). The level of 5-methylcytosine depends on the enzyme activity of DNA methyltransferase, which was found to decrease by 50% in senescent cells (12). Interestingly, stabilization of DNA methyltransferase activity and hypermethylation of CpG islands occurred during the extended life span (precrisis) of SV40-infected human fibroblasts and thus preceded the establishment of immortal cell lines (12, 13). These observations suggested the possibility that exposure of the two immortal LF lines to a demethylating agent could revert their immortal phenotype.

In a first series of experiments, sparse MDAH041 and MDAH087 cultures were exposed for 6 days to 1 μM 5-aza-CdR. Whereas the untreated control cultures were confluent after 5 days, the 5-aza-CdR-treated cultures of both cell lines never reached confluency. The growth-arrested cells had the enlarged, flat morphology of senescent cells, contained lipofuscin granules, and showed senescence-associated β-galactosidase activity at pH 6, a biomarker for senescence (Fig. 5A; Ref. 14).

The 5-aza-deoxycytidine-induced growth arrest was also evident from the fraction of cells in S phase compared with that of untreated control cells (Fig. 6). MDAH041 and MDAH087 cells were treated with 5-aza-CdR as described in "Materials and Methods" and assayed for DNA content on days 11 and 13, respectively, using propidium iodide staining and FACS analysis. The fraction of cells in S phase was reduced from 14.7 to 2.4% in MDAH041 cells and from 10.8 to 2.2% in MDAH087 cells. Bromodeoxyuridine incorporation was not used in this FACS analysis because the strong autofluorescence of the lipofuscin granules in senescent cells shifted the cells out of the cell cycle-specific quadrants, making quantitation of the results meaningless. Autofluorescence of the senesced cells may also explain the residual cells in S phase, because no mitoses were seen in the growth-arrested cultures.

To determine whether one or both of the cyclin-dependent kinase inhibitors known to be expressed in senescent cells, p16INK4a and p21CIP1, had been activated, we performed an immunoblot analysis of cell lysates from 5-aza-CdR-treated cells using antibodies to p16INK4a and p21CIP1. As seen in Fig. 2a (Lanes 1, 3, 5, and 7) and Fig. 1a (Lanes 5 and 6), lysates from 5-aza-CdR-treated MDAH041 and
MDAHO87 cells showed a greatly increased expression of p16INK4a protein, whereas no p16INK4a protein could be detected in the untreated control cultures (Fig. 2a, Lanes 2, 4, 6, and 8, and Fig. 1a, Lanes 7 and 8). In contrast, the level of p21 protein, which was low in MDAHO41 and undetectable in MDAHO87 cells, was not elevated by exposure to 5-aza-CdR (Fig. 2b, Lanes 1 and 3, and Fig. 1b, Lanes 5 and 6). These results show that activation of the expression of p16INK4a by the demethylating agent 5-aza-CdR is associated with, although not necessarily causally related to, the induction of senescence in immortal LF cells. The induction of senescence occurred without the coexpression of the p21CIP1 gene.

To confirm that the expression of p16INK4a after 5-aza-CdR treatment of MDAHO41 and MDAHO87 cells was in fact due to demethylation of a methylated 5’ CpG island of p16INK4a, its methylation status in the two lines before and after 5-aza-CdR treatment was analyzed by methylation-specific PCR (15). As shown in Fig. 7a, the 5’ CpG island of p16INK4a was methylated in both MDAHO41 cells (Lane 8M) and MDAHO87 cells (Lane 11M) before 5-aza-CdR treatment. Lane 6U shows as a control an unmethylated 5’ CpG island of p16INK4a of a human diploid fibroblast line at an early passage. Fig. 7b shows a partial demethylation of the 5’ CpG island in MDAHO41 cells (Lanes 3U and 5U 4 and 6 days after 5-aza-CdR treatment) and in MDAHO87 cells (Lanes 9U and 11U 4 and 20 days after 5-aza-CdR treatment). The data suggest a slight increase of the demethylation with increasing time after the 5-aza-CdR treatment.

Introduction of p16INK4a cDNA Induces Senescence in MDAHO41 and MDAHO87 Cells. Because induction of senescence by demethylation was accompanied by induction of p16INK4a expression, we tested whether the infection of MDAHO41 and MDAHO87 cells with a retrovirus encoding p16INK4a cDNA was sufficient to induce senescence. Like the 5aza-CdR-treated cultures, the infected MDAHO41 and MDAHO87 cultures ceased proliferation and never became confluent. The cells had the same enlarged, flat morphology as the 5-aza-CdR-treated cells and showed lipofuscin granules and senescence-associated β-galactosidase activity at pH6 (Fig. 5C). In contrast, control cultures became confluent and maintained a normal cell morphology (Fig. 5D). The expression of the introduced p16INK4a cDNA was verified by immunoblot analysis of the infected MDAHO41 cells (Fig. 1a, Lanes 1 and 2). The level of p16INK4a protein was similar to that induced by 5-aza-CdR (Fig. 1a, Lanes 5 and 6). No p16INK4a protein was present in the cells infected with the empty vector (Fig. 1a, Lanes 3 and 4). As with 5-aza-CdR, the senescence induced by introduction of the p16INK4a cDNA into MDAHO41 cells was not accompanied by changes in expression of the endogenous p21CIP1 protein (Fig. 1b, Lanes 1 and 2). It should be mentioned that Medcalf et al. (16) observed that fibroblasts derived from LF patients often lose the wild-type p53 allele upon culturing and show barely detectable p21CIP1 expression but nevertheless senesce normally.

Introduction of p21CIP1 cDNA Induces Senescence in Lines MDAHO41 and MDAHO87. Both p16INK4a and p21CIP1 are expressed in senescent normal human fibroblasts (17). Therefore, we tested whether introduction of p21CIP1 cDNA would also induce senescence in the immortal LF cells. Both MDAHO41 and MDAHO87 cultures were infected with a retrovirus expressing p21CIP1 cDNA. The infected cultures of both cell lines never reached confluency, and the cells showed all of the characteristics of senescent cells (Fig. 5E). In contrast, cells infected with virus encoding the empty vector showed normal growth properties and cell morphology (Fig. 5F). The expression of p21CIP1 protein was verified by immunoblot analysis of a lysate of senescent MDAHO41 cells that had been infected with virus expressing p21CIP1 cDNA (Fig. 1b, Lane 9). Only small amounts of p21CIP1 protein were detected in the lysate of cells infected with virus encoding the empty vector (Fig. 1b, Lane 10). Furthermore, the senescence induced by p21CIP1 occurred in the absence of endogenous p16INK4a expression (Fig. 1a, Lane 9).

Discussion
We have shown that induction of senescence in immortal LF fibroblast lines can occur by independent pathways: (a) by demethylation-dependent pathways in which the expression of p16INK4a is induced; and (b) by demethylation-independent pathways involving the expression of p21CIP1. The experiments do not exclude the possibility that demethylation-independent pathways exist that induce the expression of p16INK4a as well.

During the replicative senescence of normal human fibroblasts, the expression of both p21CIP1 and p16INK4a in-
crease dramatically. The increase is sequential; p21^{CIP1} expression increases when the majority of cells have lost their growth potential, whereas p16^{INK4a} expression increases in the terminal stages of senescence when all cells have lost their growth potential (17). A role for p16^{INK4a} in the senescence of normal human fibroblasts has only been recognized recently (17–19). It was shown that p16^{INK4a}, and not p21^{CIP1}, binds in the late stages of senescence to both CDK4 and CDK6 kinases (17).

We have demonstrated that the treatment of human immortal fibroblasts with 5-aza-CdR leads to a partial demethylation of the CpG island of p16^{INK4a} and to the expression of the gene. During replicative senescence of human fibroblasts the decrease in the methylation of the p16^{INK4a} gene may be a consequence of the decrease in methyltransferase activity. Interestingly, Chuang et al. (20) have found that expression of methyltransferase and p21^{CIP1} are inversely related due to mutually exclusive complex formation with proliferating cell nuclear antigen. The activation of p21 precedes the activation of p16^{INK4a} in replicative senescence and is most likely due to the observed increase in the transcriptional activity of p53 (21). This activation of p21^{CIP1} could lead to the down-regulation of methyltransferase and explain the activation of p16 late during the replicative senescence.

Finally, our results suggest that the telomere-mediated clock and the demethylation-associated clock act independently of each other. It seems likely that the telomere-medi-
ated clock becomes active in cells in which telomeres shorten to a critical length due to an extended life span induced by the loss of expression of p53, p21<sup>CIP1</sup>, p16<sup>INK4a</sup>, or other suppressors.

Materials and Methods

Cell Culture. The two immortal LF fibroblast lines MDAH041 and MDAH087 were kind gifts from M. A. Tainsky (8). The cells were cultured at 37°C in 10% humidified CO<sub>2</sub> in DMEM (Sigma Chemical Co.) supplemented with 10% FBS (HyClone), 50 units/ml penicillin, and 100 μg/ml streptomycin.

Treatment with 5-aza-CdR. On days 1, 3, and 5, fresh medium supplemented with 1 μM 5-aza-CdR was added to the cultures. After day 6, the cells were kept in regular medium without 5aza-dCR. The 5-aza-dCR-treated cultures never reached confluency.

Infection with pCLp16SN-, pCLp21SN-, and pCLXSN-Virus. cDNAs of p16<sup>INK4a</sup> and p21<sup>CIP1</sup> were kindly given to us by David Beach. The cDNAs were inserted at restriction sites EcoRI/Xhol (p16) and EcoRI (p21) in the pCLXSN vector (22). Two × 10<sup>6</sup> cells/100-mm dish of the adenovirus-transformed human embryo kidney cell line, 293 (23), were cotransfected with 20 μg of pCLp16SN-, pCLp21SN-, or pCLXSN-DNA and 20 mg of a pCLampico packaging construct by a modification of the calcium-phosphate method (24). After 6 h, a 15% glycerol shock was applied for 1 min, the cells were washed, and fresh medium was added. Fresh medium was again added after 18 h, and the supernatants were harvested 24 and 48 h later. The titers varied between 1 and 3 × 10<sup>5</sup> G418 colony-forming units/ml for the pCLp16SN and pCLp21SN viruses and between 3 × 10<sup>5</sup> and 3 × 10<sup>4</sup> G418 colony-forming units/ml for the vector-only (pCLXSN) virus.

Four × 10<sup>5</sup> MDAH041 or MDAH087 cells/50-mm dish were infected four times within 24 h; after 48 h, the cells were split 1:6 and cultured in the presence of 200 μg of G418/ml.

Senescence-associated β-Galactosidase Staining. The cells were tested for SA-β-galactosidase activity at pH 6.0, as described by Dimri et al. (14). Briefly, the cells were washed with PBS, fixed for 3 min with 2% formaldehyde/0.2% glutaraldehyde, washed with PBS, and incubated overnight at 37°C in fresh 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution (1 mg/ml) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Fisher Biotech; 0.5 mM KI3Fe(CN)<sub>6</sub>, 0.5 mM K4Fe(CN)<sub>6</sub>, 150 mM NaCl, 2 mM MgCl<sub>2</sub> in 40 mM citric acid/sodium phosphate, pH 6.0).

Immunoprecipitation and Immunoblotting. Cells were lysed in 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% NP-40, 500 μM phenylmethylsulfonyl fluoride, and 1% aprotinin. The lysates from equivalent numbers of cells were centrifuged for 10 min at 10,000 rpm and precleared by incubation at 4°C for 30 min with protein A beads (Repligen). The lysates were incubated with 1 μl of the appropriate polyclonal antibody (Pharmingen; α-human p16 or α-human p21) overnight at 4°C, collected on protein A beads (preblocked with 3% nonfat powdered milk), washed four times with lysis buffer, boiled for 2 min in sample buffer, electrophoresed on a 15% SDS-polyacrylamide gel, and transferred to nitrocellulose. The blots were blocked with 5% nonfat dry milk and incubated overnight at 4°C with the appropriate polyclonal antibody (above) diluted 1:500. The peroxidase-conjugated donkey anti-rabbit IgG (Amersham) was incubated with the blot for 1 h at room temperature and detected by enhanced chemiluminescence (Amersham).

FACS Analysis. Cells were prepared and labeled with propidium iodide using standard methods for cell cycle analysis. Flow cytometric analysis was carried out on a fluorescence-activated cell scanner (FACScan; Becton Dickinson) using Lysis II software.

Telomerase Assay. Cytoplasmic S-100 extracts were prepared by a modification of the method of Counter et al. (25). Adherent cells were washed twice with ice-cold PBS, scraped off the plates in cold PBS, and pelleted at 2500 rpm for 8 min. Pellets were resuspended in 0.5 ml 10-cm plate of 2× Hypobuffer [1× hypobuffer: 10 mM Tris-HCl (pH 8.3), 3 mM KCI, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 10 μg pepstatin, and 100 units/ml recombinant RNase; Promega], transferred to microfuge tubes, and centrifuged at 10,000 rpm (8200 × g) for 10 min at 4°C. The pellets were resuspended in 100–150 μl 10-cm plate of 1× hypobuffer. The pellets were allowed to swell at 4°C for 15–30 min and then were lysed by 5–10 passages through a 25-gauge needle. After an additional 15-min incubation at 4°C, nuclei were removed by two successive centrifugations at 4°C at 12,000 rpm (11,900 × g) for 20–30 min each. Glyceraldehyde was added to the supernatant fraction at a concentration of 10%, and aliquots were flash frozen and stored at −70°C.

The telomerase assay is a modification of the method of Morin (26), developed at the Salk Institute by Dr. Oleg Glebov. The S-100 extracts...
were diluted in 1× hypobuffer + 10% glycerol to contain 0.001–14 μg of protein in 20 μl, then mixed with a 20-μl extension mix containing 1× hypobuffer, 5–10 μg BSA, 0.1 mM dATP, dGTP, and dTTP, 0.25 mM spermine, 0.025 mM spermidine, 0.0175 mg/ml oligonucleotide 5′-CGA/CAT/GCA/TCG/AGA/GTT-3′. The extension reactions were incubated 1 h at 30°C, followed by the addition of 130 μl of 0.01 M Tris-HCl (pH 8.0), 0.001 M 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid hydrate (pH 8.0), 0.5% SDS, and 1 mg/ml proteinase K, with further incubation at 37°C for 1 h. The reactions were then adjusted to 0.2 mM salt with LiCl or sodium acetate, then phenol extracted, chloroform extracted, and ethanol precipitated overnight at −20°C after the addition of 0.1 mg/ml yeast RNA carrier. Centrifugation at 12,000 rpm for 30–60 min at 4°C resulted in pellets that were dried and resuspended in 50 μl of Milli-Q water.

The PCR mixture for the amplification contained in a final volume of 20 μl: 2 μl extension reaction in water, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, dATP, dGTP, dTTP (each at 0.1 mM), 2.5 mM dCTP, 0.05 mM [32P]dCTP, 0.04 unit/μl Ampli-Taq polymerase (Perkin-Elmer), and primers each at 5 μM. The primers used were 5′-CGA/CAT/GCA/TCG/AGA/GTT-3′ and 5′-GCA/CTT-GCC-TAA-CCC-TAA-3′. The amplification conditions were: 4 min at 94°C; 30 cycles (30 s at 94°C, 1 min at 55°C, and 1 min at 72°C), followed by 5 min at 72°C. Three μl of the amplification products were mixed with formamide loading dyes, denatured at 95°C for 2 min, then resolved on 8% Long Ranger (FMC Bioproducts) acrylamide–7 M urea denaturing gels in 0.6× TBE (89 mM Tris-base, 89 mM boric acid, 2.5 mM Na2EDTA, pH 8.3) at 33 W until the BPE (0.025% bromphenol blue; 0.01% w/EDTA; 1% SDS; 6.75% glycerol) was 5 cm from the bottom of the gel. Dried gels were exposed to Kodak BioMaxMR film at room temperature for 3–14 days.

Determination of Telomere Lengths. DNA isolation was accomplished by standard methods. Briefly, high molecular weight genomic DNA was isolated from cultured cells by SDS lysis and proteinase K digestion followed by two rounds of phenol and chloroform extractions interspersed by dialysis against 0.01 M Tris (pH 8.0), 0.001 M trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid hydrate, and 0.2 M LiCl, followed by ethanol precipitation. Purified DNA was digested with restriction enzymes HinfI and RsaI and resolved on a 0.7% Seakem GTG agarose (FMC Bioproducts) gel in TAE buffer (40 mM Tris-base, 20 mM acetic acid, 2 mM NaN3, EDTA, pH 8.1). After visualizing the DNA, the gel was dried onto Gel Bond film (FMC Bioproducts) and treated according to Purrello and Balazs (27) with SSPE (0.18 M NaCl; 10 mM Na2PO4, pH 7.7; 1 mM EDTA) used in place of SSCP (0.15 M NaCl; 0.015 M sodium citrate; 0.025 M phosphate, pH 7.0). The TRFs were detected by hybridization at 60°C to a specific primer (AATCCCl2-labeled 800-bp fragment containing TTAGGG repeats (a gift of Titia de Lange, Rockefeller University, New York, NY). The gel was stringently washed at 60–64°C, dried, and exposed to Kodak XAR-5 film using an intensifying screen at −70°C.

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References